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Deletions near the N-terminus of HIV-1 Rev reduce RNA binding affinity and dominantly interfere with Rev function irrespective of the RNA target

K.-S. Jeong, Y.-S. Nam, and **S. Venkatesan**

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, U.S.A.

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Summary. The contributions of the near N-terminal residues of Rev protein of HIV were investigated by analyzing N-terminal deletions of Rev in the context of a Rev/MS-C fusion protein that can bind and activate both the Rev responsive element (RRE) and the MS2 phage translational operator RNAs. Rev/MS-C fusion proteins deleted for residues 3–19 of Rev retained *trans*-activation potential for both RRE and MS2 targets. Coincidentally, peptides spanning residues 17–87 or 22–85 were functionally competent for *trans*-activation of RRE containing HIV-1 *gag* mRNA. Deletion of residues 18–24 of Rev in the Rev/MS-C fusion protein abolished the activation potential for both RRE and MS2 targets, although this mutant was competent for specific RNA binding, protein multimerization, and nuclear and nucleolar localization. Four mutants dominantly interfering with Rev activation of RRE were mapped near the N-terminus of Rev; (i) between residues 18 and 24, (ii) 25–34, (iii) 43–50, and (iv) 51–60. Of these, the mutant lacking residues 18–24 was a novel *trans*-dominant inhibitor of Rev and Rev/MS-C for activation of RRE or MS2 RNA, while the oligomerization domain mutants mapping between residues 25–34 or 51–60 inhibited the activation of RRE rather than MS2 RNA.

Introduction

In the lifecycle of HIV, the Rev protein regulates the temporal switch from the early regulatory to the late replicative phase [1, 12, 28]. The Rev regulatory protein of HIV is a basic nuclear protein that concentrates in the nucleoli [6, 8, 15, 20, 24, 27, 35, 43, 48, 59, 62] and activates the viral RNAs, by binding to a highly structured RRE (Rev Responsive Element) RNA [9, 19, 21, 23, 36, 37, 49, 63]. In the presence of Rev, RRE containing viral RNAs are transported out of the nucleus and translated into viral structural proteins or packaged in the nascent

viral particles. The 116 amino-acid Rev regulatory protein of HIV is a modular protein with N-terminal arginine rich RNA binding motif between residues 35 and 50 [6, 20, 22, 31, 37, 54, 63, 64], and a relatively acidic, leucine rich effector domain between residues 75–93 [25, 39, 42, 59, 60] which interacts with the nuclear export factor, CRM1 [14, 17, 45, 51], nuclear pore associated proteins [5, 16], and nuclear eIF-5A [50]. The N-terminal basic domain is also required for nuclear (NLS) and nucleolar (NOS) localization and flanking this domain are sequence determinants for protein oligomerization [26, 33, 34, 38, 44, 64]. Direct interaction of Rev with the RRE RNA is not an absolute requirement for trans-activation; Rev can activate heterologous RNAs if it is directed to it by another RNA binding protein [41, 58].

Rev domains critical for RNA binding, nuclear and nucleolar localization, and protein oligomerization have been genetically and biochemically mapped between residues 25 and 66 [6, 20, 22, 25, 26, 33, 34, 37–39, 42, 44, 59, 60, 64]. Although the leucine rich effector motif is necessary for Rev mediated nuclear export of RRE transcripts in vivo [5, 13, 14, 17, 45, 51–53], a minimal basic peptide spanning residues 35–50 of Rev has been shown to specifically bind RRE RNA and inhibit splicing of RRE containing RNAs in vitro [7, 30, 31]. In vitro experiments with minimal basic Rev peptides have identified hierarchical contact points on the RNA for arginine forks of the peptide [29, 31]. However, with respect to Rev, as with other RNA binding proteins, the issues of RNA- binding affinity vs. binding specificity have not been explored sufficiently. It has been largely presumed that sequence immediately upstream of the core RNA binding site between positions 35–50 is needed for discrimination of RRE from non-specific RNAs [2, 3, 54, 55]. Subsequent studies using a Rev domain peptides and deletion mutants have shown that N-terminal sequence of Rev discriminates RRE form non-specific RNA [10].

In this paper, we have defined the minimal structural requirements for a functionally competent Rev using mutants that deleted portions of the N-terminal region of Rev in the context a Rev/MS-C fusion protein that could be targeted to RRE or MS2 RNA. Rev truncated to between residues 19 and 87 still retained most of the Rev function. We complemented these studies with analysis of the RNA binding and *trans*-activation potential of a family of Rev peptides. We show that under conditions of stringent specificity, a minimal Rev peptide encompassing residues 22–85 and containing the effector motif retained fidelity of Rev function in vivo albeit with reduced in vitro binding affinity for RRE RNAs. More extensive deletions in the minimal peptide resulted in a further loss of affinity that may have been interpreted previously as lost specificity. We further show that a Rev mutant that deleted residues 18–24 in the context of the Rev/MS-C fusion protein had a null phenotype for RRE and MS2 targets. This deletion mutant was also a novel *trans*-dominant inhibitor of Rev function, distinct from other inhibitory mutants mapping to the NLS/NOS and oligomerization domains of Rev. Other mutants lacking the multimerization domain(s) of Rev repressed Rev protein(s) targeted to RRE or heterologous MS2 RNA in a differential manner. The mechanistic implications of these findings are discussed.

Materials and methods

Expression plasmids

Construction of the HIV-1 LTR linked GAG expression plasmids used in this study has been described before [23]. RRE refers to a GAG expression plasmid containing the 244 nt HIV-1 RRE sequence. In RREZ-MS, the Rev responsive stem loop II sequence of RRE was exchanged for the phage MS2 translational operator sequence [58]. *gag*-TAR and *gag*-VAI were constructed by exchanging the RRE sequence in the HIV-LTR linked *gag* expression plasmid for the PCR amplified, TAR and adeno virus VA I DNAs [46] respectively. HTLV-I RexRe containing HIV-I GAG expression plasmid was a gift from George Pavlakis of NCI/FCRDC.

HIV-1 Rev was expressed either from the TAT responsive HIV-1 LTR or from the constitutive RSV-LTR [23]. Rev/MS-C denotes a RSV LTR linked expression plasmid encoding a tandem fusion of Rev and MS-C ORFs [58]. The various mutants were engineered into the Rev/MS-C ORF by two step overlap PCR strategy and were cloned at the XbaI site of an RSV-LTR linked plasmid, pRSV.5 [58]. The mutants were verified by DNA sequencing and the individual inserts were PCR amplified with primers containing T7 polymerase promoter and terminator tags at the 5′ and 3′ respectively. The PCR fragments were transcribed and translated in vitro using a coupled system (Promega Corp.) using [^{35}S] methionine to label the proteins. The various Rev/MS-C derivatives were translated into fusion proteins of the expected size, immuno-reacting with anti-Rev antiserum. Rex was expressed from a RSV-LTR linked Rex expression plasmid that was a gift from George Pavlakis of NCI/FCRDC.

Peptide synthesis and characterization

Peptides were synthesized by the Peptide Synthesis and Analysis Unit of NIAID. Peptides were purified by two cycles of reversed phase HPLC on C18 columns and their purity checked by time-of-flight mass spectrometry on a Vestec LD-TOF Mass Spectrometer. Amino-acid composition was analyzed by use of a 6300 Beckman Analyzer. Each peptide was subjected to automated N-terminal micro-sequencing by Edman degradation over 30 cycles. To minimize oxidation, peptides were stored in a lyophilized state under liquid N_2. Lyophilized peptides were dissolved in PBS, and then adjusted to 80% DMSO in PBS, or in DMF and adjusted to 50% DMF in PBS. Since most of the Rev peptides had a cysteine residue at or near the C-terminus, fresh solutions of peptides were reduced by reaction with $NaBH_4$ as described [18]. To prevent dimerization through sulfhydryl groups, peptides were reacted with 50 fold molar excess of iodo-acetamide in 50% DMF in PBS and purified by gel-filtration chromatography on P2 columns (Pharmacia Upjohn). Loss of or preservation of free thiol groups was quantified by reacting with DTNB (Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid)) and reading the optical density at 412 nm.

E. coli *expression of Rev/MS-C fusion proteins*

Rev and Rev/MS-C protein derivatives were expressed in *E. coli* as fusion proteins, linked to the C-terminus of *E. coli* maltose binding protein (MBP) from an IPTG inducible β-galactosidase promoter using a commercial kit (New England Biolabs, Beverly, MA) essentially as described before [58]. Recombinant protein expression was induced by IPTG (1 mM) at $OD_{600} = 0.3$ followed by 4 h incubation in at 30 °C. Bacteria were disrupted by a French Press at 10,000 psi in a buffer (10 ml per packed cell volume) containing 50 mM Tris-HCl pH 7.8, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM 4-(2-amino-ethyl) benzene-sulfonyl-fluoride (AEBSF), aprotinin (2 μg/ml), leupeptin (1 μg/ml) and pepstatin (2 μg/ml). Extracts were centrifuged at 25,000 × g for 15 min to collect pellets enriched

for inclusion bodies. MBP tagged fusion proteins were purified by affinity chromatography of proteins extracted from inclusion bodies on amylose resin as described by the manufacturer. Procedures pertinent to MBP tag excision by Factor Xa proteolysis and purification of the MBP free proteins were according to the manufacturer (New England Biolabs Corp). Completion of Factor Xa digestion was monitored by SDS/PAGE and immuno-blotting with rabbit anti-Rev antiserum. After removing the residual maltose by hydroxyl-apatite chromatography, MBP and undigested MBP fusion proteins were removed by binding to amylose resin. The respective Rev and Rev/MS-C proteins in the flow-through fraction from the amylose chromatography were bound to carboxy-methyl cellulose, CM 52 or phosphocellulose, P11 (Whatman Corp), and batch eluted with NaCl. Protein concentrations were measured by Bio-Rad Bradford assay. Fractions were monitored for Rev by dot immunoblotting and phosphor-imager quantitation (Molecular Dynamics, OR). The final eluates were pooled, concentrated and dialyzed against PBS using Centricon 10 columns (Amicon Corp).

RNA synthesis and protein binding

T7 RNA polymerase initiated transcription of various templates and RNA purification have been described [23]. Typically, the template DNAs were prepared by PCR amplification of the respective genomes using 5′ T7 promoter tagged primers. Smaller templates were prepared by annealing the single stranded DNAs and purifying the ds DNAs by PAGE after nuclease S1 digestion of residual ss DNAs. For the experiments described in the manuscript, the following templates were used: RRE, 244 bp DNA corresponding to nts 7749-7992 of NL4-3 HIV genome; RREZ, RRE DNA deleted for the Rev responsive SLIIB domain of 64 bp; RREZ-MS, 19 bp MS2 translational operator stem-loop sequence in place of the SLIIB domain of RRE; TAR, 52 bp TAR DNA fragment; and MS2 RNA, 19 bp MS2 operator DNA. RNA:protein binding was evaluated by electrophoretic mobility shift assay (EMSA) and nitrocellulose filter binding. Binding conditions have been described before [23]. In general, protein or peptides and heparin (5 μg) in HEPES binding buffer (20 mM Hepes-KOH, pH 7.9, 62 mM KCl, 0.15 mM DTT, and 6% glycerol) was pre-incubated at 30 °C for 10 min before addition of ^{32}P-labeled RNA and continued incubation for 10 min at 30 °C. Specific changes such as varying the amounts of peptide or protein, addition of competitor RNAs are described in the respective figure legends. Samples were electrophoresed at 30 mA at 4 °C 5% native poly-acrylamide gel in 0.5 × TBE. Radioactivity was visualized by auto-radiography of dried gels. From preliminary titration experiments, at protein:RNA ratio of about 5:1, approximately 90% of RNA was converted into an RNA-protein complex. Rev protein used in these studies expressed in *E. coli* and purified to near homogeneity [61]. Filter-binding assays were done as described [23]. Typically, ∼200 fmol (4–5 × 10^4 dpm) of ^{32}P-UMP labeled RRE or RREZ-MS RNA was incubated at 25 °C for 10 min in 10 μl of binding buffer (20 mM TRIS-HCl, pH 7.5, 0.05 M KCl, 1 mM DTT, 5 mM spermidine, 20 μg BSA, and 2 μg of yeast tRNA) with increasing concentrations of the respective REV fusion proteins purified by affinity chromatography. The samples were filtered through pre-wetted 25 mm nitrocellulose filters (0.45 μ pore size, Schleicher & Schuell) at a flow-rate of 5 μl/sec. Filters were rinsed twice with 0.75 ml of binding buffer (without BSA, spermidine or tRNA), air dried and the bound radioactivity determined.

Transient expression assay

The general protocols have been described before [23, 58]. For DNA transfections 2 × 10^6 HeLa or Cos-7 cells were electroporated at 300 V and 250 μF in a Bio-Rad electroporator with the individual *gag* plasmids (5 μg) and 2 μg of pHIV-TAT (pSV40 TAT for Cos cells)

with the indicated Rev or the Rev/MS coat protein fusion protein plasmids. For expression of Rev, 2.5 or 5 μg of HIV-1 LTR or RSV-LTR linked plasmid was used. HIV-1 LTR linked CAT or luciferase (LUC) (2 μg) plasmid was added to normalize for constant LTR transcription in transfections using HIV-LTR linked *gag* plasmids. CMV-CAT or RSVLTR-LUC was used for normalizing other transfections. CAT expression was quantified by a commercial CAT ELISA kit (Boehringer Mannheim), and LUC assay was by use of a commercial kit (Promega Corp) designed for microplate luminometer. Aliquots of transfected cells were removed at 48 h and used for immune-detection of Rev and Rev/MS fusion proteins (see below). For immuno-fluorescence experiments, an aliquot of cells each was plated on cover-slips immediately after electroporation and processed for of Rev and Rev/MS fusion proteins. GAG expression was quantified by p24 ELISA of cell extracts (Coulter Diagnostics). For quantifying the GAG or CAT ELISA values, extracts of cells expressing wt Rev and gag RNA and cells expressing CMV CAT extracts were serially diluted to obtain GAG or CAT ELISA OD units of between 1 and 2. Under these conditions, positive control readouts for GAG or CAT expression in different experiments varied between 200 and 300 serial dilutions. The cut-off value (recommended by the manufacturer) for the negative response was at ELISA readings that fell below 10% of the values for the respective RRE/REV or CMV-CAT values. ELISA readouts of undiluted or serially diluted extracts of non-transfected cells fell below the 10% values of positive control cells. GAG expression levels were normalized to constant values of CAT or LUC activity from HIV LTR linked CAT [58]. For introducing of proteins or peptides, Cos-7 cells were more efficient. Optimal electroporation conditions for peptide uptake were determined by immuno-blotting and immuno-fluorescence microscopy. To improve the intra-cellular stability of the peptides, chloroquine (60 μM) was routinely added after electroporation.

Immunological methods

Immuno-blotting for Rev and indirect immuno-fluorescence detection of Rev, Rev peptides and fusion proteins in transfected cells have been described in detail before [58]. Cell extracts were processed for SDS/PAGE as described except that for resolving peptides, 17.5% poly-acrylamide gels were used. For immuno-detection of peptides, commercial (Intracel Corp., Issaquah, WA) rabbit polyclonal anti-Rev antiserum was used routinely to avoid the loss of detection by monoclonal antibodies against absent or masked epitopes. The filters were then incubated with the appropriate species specific second antibody tagged with horseradish peroxidase. And the immuno-reactive bands were visualized by means of a commercial chemi-luminescence protocol (Amersham Corp) and quantified by a commercial phosphor-imager scanner (Molecular Dynamics, OR). For indirect immuno-fluorescence microscopy, permeabilized and fixed cells on cover-slips were treated with rabbit polyclonal anti-Rev anti-serum (1:200 dilution) or commercial (Intracel Corp., Issaquah, WA) anti-Rev mouse monoclonal (epitope mapped to residues 96–110) antibody (1:500 dilution) in 0.2% BSA for 4 h at RT. This was followed by a 1 h reaction with fluorescein labeled goat anti-rabbit Fab fragment (1:500) or donkey anti-mouse antiserum (1:200) in 0.2% BSA in PBS.

Protein cross-linking

For cross-linking experiments, the thiol-reversible NHS-ester cross-linker, dithiobis-(sulfosuccimidyl propionate) (DTSSP) was used. Individual recombinant fusion proteins, purified after Factor Xa cleavage and MBP removal were adjusted to constant Rev equivalents in 20 μl of PBS and reacted with 2 μl of freshly dissolved DTSSP (50 mg/ml in DMSO) for 1 h at 0–4 °C. Reactions were terminated by addition of $1/10^{th}$ volume of a solution containing glycine (1 M) and ethanolamine (1 M), and the incubation was continued for another

hour. Reactions were denatured by boiling in 1% SDS and electrophoresed on SDS gels without reducing agents. For disulfide reduction, reactions were denatured by boiling in 1% SDS containing 1 M DTT. Following SDS/PAGE, the gel was blotted to PVDF membrane which was processed for immuno-blotting with rabbit anti-Rev antibody and chemiluminescence detection of immuno-reactive bands.

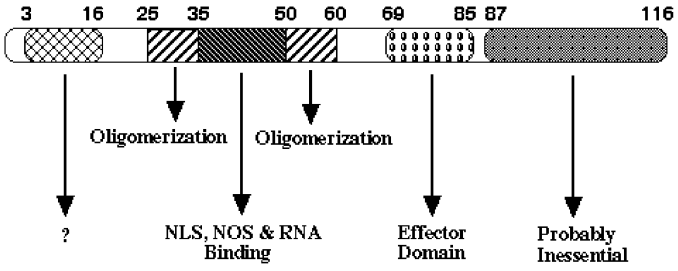
Cyto-toxicity and permeability characteristics constrained the selection to a few Cross-linking reagents for in vivo work, and DTBP proved to be the most consistent. Transfectants on 60 mm dishes were rinsed several times with ice cold PBS, and the monolayers were dislodged by treatment with PBS containing 5 mM EDTA. The cells were collected by centrifugation, rinsed three times with ice cold PBS and suspended in 0.2 ml of PBS containing DTBP (1 mg/ml), diluted from a freshly dissolved stock solution (50 mg/ml in DMSO). The cell suspension was incubated at 4 °C for 1–2 h and the excess DTBP was neutralized by addition of $1/10^{th}$ volume of PBS containing glycine (1 M) and ethanolamine (1 M), and continued incubation for another hour. The cells were harvested by centrifugation, and disrupted in a buffer containing by two cycles of freeze-thaw in 50 μl of a buffer containing 50 mM Tris HCl, pH 7.4, 0.5% NP-40 and 0.5% of CHAPS. The cell extracts were adjusted to 1 mM AEBSF, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 2 μg/ml of pepstatin and incubated at 37 °C for 15 min with a mixture of pancreatic ribonuclease A (2 μg/ml) and 10 units of RQ DNAse (Promega Biotec). The digestion was stopped by the addition of an equal volume of a buffer containing 50 mM TRIS HCl, pH 7.4, 4% SDS, and 12% (w/v) glycerol, and heating at 95 °C for 5 min. The samples were subjected to SDS/PAGE under non-reducing conditions followed by immuno-blotting.

Results

Excision of residues between the N-terminus and the 5′ oligomerization domain of Rev is compatible with RNA binding in vitro and multimerization in vitro and in vivo

The schematic diagram (Fig. 1) of the functional domains of Rev with the amino acid sequence Rev serves as a frame of reference for the different mutants and peptides used in this study. Previously it was shown that N-terminal truncations of Rev to the 17^{th} or 22^{nd} residue resulted in a loss of discrimination of RRE from other non-specific RNAs [10]. We inquired whether similar lesions in the context of Rev were disruptive for RNA binding, protein multimerization and *trans*-activation. To distinguish the negative effects of poor RRE RNA binding from other functional defects, we engineered a nested set of deletions near Rev N-terminus in the context of the Rev/MS-C fusion protein. The relative potential of mutants for in vivo activation of *gag* mRNAs incorporating RRE or MS2 operator targets, could then be used to identify defects other than poor RRE RNA binding.

In vitro RNA binding properties of various Rev deletions was evaluated. For this purpose, the deletions engineered into the Rev/MS-C chimera were expressed in E. coli tagged C-terminally with the maltose binding protein (MBP). The various MBP tagged Rev/MS-C proteins were purified from bacterial lysates by affinity chromatography on amylose resin. As shown by the electrophoretic profile in Fig. 2A, nearly homogeneous preparations of various Rev N-terminal deletion mutants were obtained in this manner. Rev content in the various fusion proteins

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                                                          25
1   M A G R S G D S D E D L L K A V R L I K F L Y Q S N P P P N  30
                  35                                  50
31  P E G T R Q A R R N R R R R W R E R Q R Q I H S I S E R I L  60
                69                                        85
61  S T Y L G R S A E P V P L Q L P P L E R L T L D C N E D C G  90

91  T S G T Q G V G S P Q I L V E S P T I L E S G A K E 116
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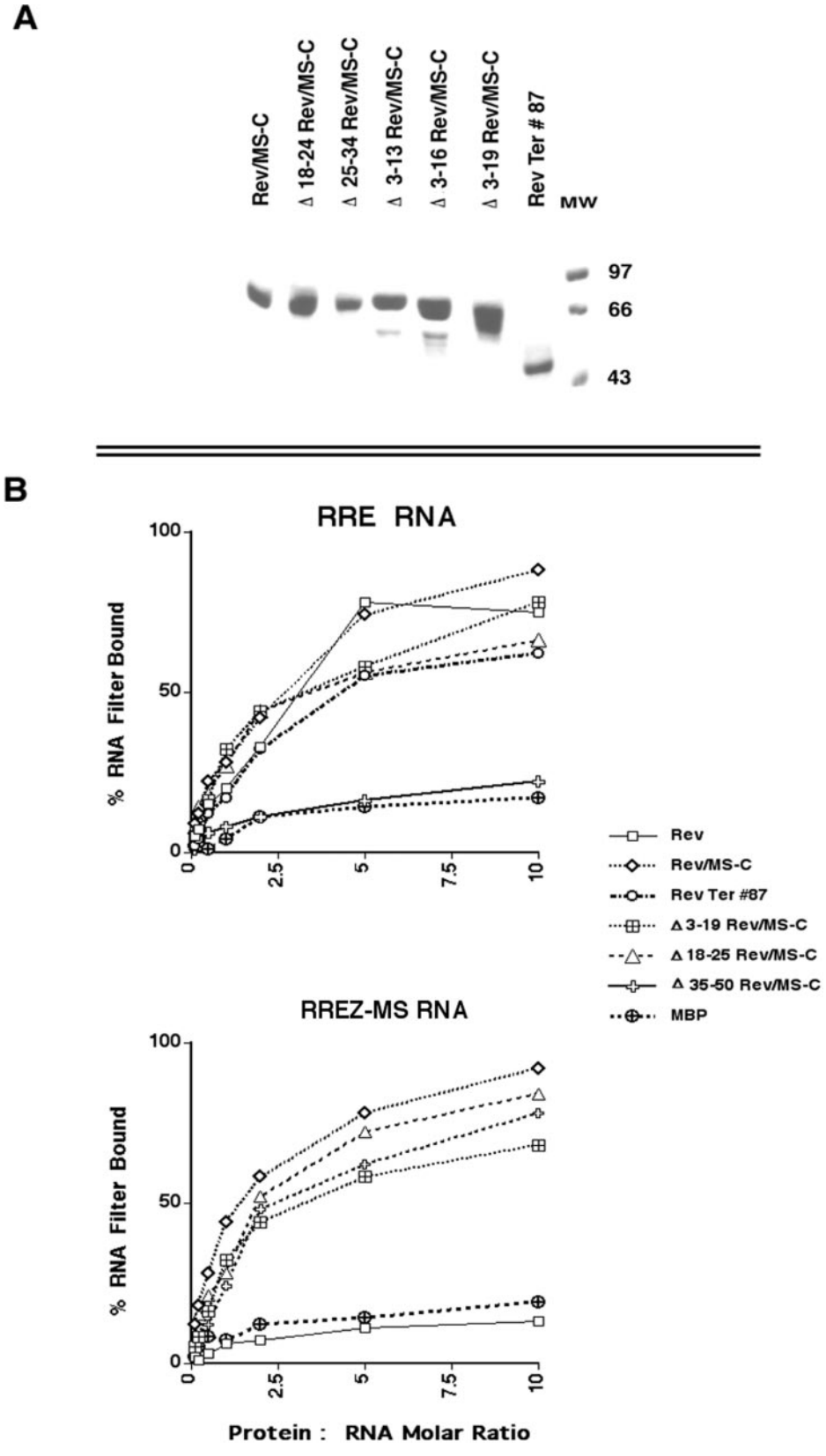
Fig. 1. Schematic diagram of the functional domains of Rev with the respective amino-acid sequence coordinates is shown at the top. Numbered amino-acid sequence of Rev shown below provides a frame of reference for the different peptides and mutants used in the study

was determined by quantitative ELISPOT assay using affinity-purified rabbit antibodies raised against purified Rev. Individual preparations were adjusted to constant Rev equivalents.

For measuring RNA binding affinity and activation response, RRE and RREZ-MS RNAs were used as the targets for Rev and MS2 coat protein binding. In RREZ-MS, the 64 nt Rev responsive core domain, SLIIB had been exchanged for the MS2 translational operator RNA. Although the Rev/MS-C fusion protein was capable of activating a non-chimeric MS2 RNA when present as a concatenated tetramer [41, 58], the RREZ/MS chimera was far more efficient in this regard, presumably because the 2° structure of MS2 RNA is well preserved in the chimera. Previous work has shown that RREZ lacking the SLIIB motif and the RREZ-MS chimera was neither bound nor activated by Rev [58]. RNA binding ability of various Rev mutants was evaluated by filter-binding assay with RRE and RREZ-MS RNA targets (Fig. 2B). At a protein to RNA stoichiometry of about 10:1, greater than 60% of the input RNA became filter bound by virtue of binding to the respective cognate protein (such as RRE and Rev). Under the same conditions, less than 15% of these two RNAs was bound by non-specific protein(s) like MBP even at a 100 fold molar excess (not shown). All the N-terminal Rev mutants except for the one that excised of the RNA binding basic domain (Δ 35/50 Rev/MS-C) bound RRE RNA relatively equivalent to wt Rev. Truncating Rev to the 87th residue did not affect RRE RNA binding. The Rev/MS-C mutant (Δ 35/50 Rev/MS-C) that deleted the RNA binding basic domain of Rev bound RREZ-MS RNA almost as


A
Rev/MS-C
Δ 18-24 Rev/MS-C
Δ 25-34 Rev/MS-C
Δ 3-13 Rev/MS-C
Δ 3-16 Rev/MS-C
Δ 3-19 Rev/MS-C
Rev Ter # 87
MW
97
66
43
B
RRE RNA
% RNA Filter Bound
100
50
0
0
2.5
5
7.5
10
Rev
Rev/MS-C
Rev Ter #87
Δ3-19 Rev/MS-C
Δ18-25 Rev/MS-C
Δ 35-50 Rev/MS-C
MBP
RREZ-MS RNA
% RNA Filter Bound
100
50
0
0
2.5
5
7.5
10
Protein : RNA Molar Ratio

well as wt Rev/MS-C, thus confirming that binding to MS2 RNA is mediated by the MS2 coat protein moiety. As expected, wt Rev failed to bind to this chimera.

The various proteins were also evaluated by EMSA for their potential to multimerize on the respective cognate RNAs (Fig. 3). For this experiment, purified Rev or Rev/MS-S derivatives devoid of MBP tags were used. Deletions (Δ 3/19 Rev/MS-C and Δ 18/24 Rev/MS-C) near the N-terminus upstream of the Rev oligomerization domain in the Rev/MS-C protein retained the potential to form higher order RNA complexes with RRE RNA (Fig. 3A I). Truncating the Rev/MS-C fusion protein in the above and related constructs (Δ 3/13 Rev Ter #87 and Δ 3/19 Rev Ter #87) to the 87th residue of Rev preserved this potential (Fig. 3A II). Removing either the N-terminal (Δ 25/34 Rev/MS-C) or the C-terminal (Δ 51/60 Rev/MS-C) multimerization motifs resulted in a corresponding loss of multimer formation (Fig. 3A II). Under the conditions of this assay, wt Rev and the 86 amino-acid truncation mutant yielded mostly dimers and higher order species with RRE as was the case also with Rev/MS-C and RREZ/MS RNA (Fig. 3B). As expected, deletion (Δ 35/50 Rev) of RNA binding domain of Rev abolished RNA complexing (Fig. 3A II). A similar pattern of RNA binding was observed with RREZ/MS RNA (Fig. 3B). N-terminal deletions of upstream of the Rev oligomerization domain (Δ 3/19 Rev/MS-C and Δ 18/24 Rev/MS-C) preserved the formation of higher order RNA complexes. Excision of either the N- (Δ 25/34 Rev/MS-C) or the C-terminal (Δ 51/60 Rev/MS-C) multimer motifs resulted in a corresponding decrease in oligomer formation.

The ability of the various mutant proteins to multimerize in vitro and in vivo was analyzed by protein cross-linking experiments. Mutants expressed in *E. coli* as MBP tagged proteins were purified by affinity chromatography and the MBP tags were removed by Factor Xa proteolysis. The Rev and Rev/MS-C fusion proteins were further purified by negative selection on amylose resin followed by batch-wise elution from carboxy-methyl cellulose (CM52) columns as described under *Experimental procedures*. Each protein was adjusted to constant Rev equivalent before reacting with the thiol reversible bi-functional chemical cross-linking reagent, DTSSP. Under these conditions, Rev/MS-C was cross-

Fig. 2. In vitro RNA binding of various Rev/MS-C derivatives expressed in *E. coli* as MBP fusion proteins. **A** Rev and Rev/MS-C MBP fusion proteins were purified as described under *Experimental Procedures*. Coomassie blue stained gel electrophoretic profile (12% SDS/PAGE gels) of affinity-purified fusion proteins is shown. **B** Nitrocellulose filter binding assay of RNA binding. Constant amounts (ca. 200 fmol or 4–5 $\times$ 10^4 dpm) of uniformly labeled wt RRE or RREZ-MS RNA were incubated with increasing concentrations of REV or various Rev-MS-C fusion proteins. All proteins other than Rev were expressed and purified as MBP tagged proteins. Individual fusion proteins (identified by symbols in the legend) were adjusted to constant Rev equivalents. Filter bound radioactive RNA (expressed as % of total input dpm) was plotted as a function of the ratio of Rev equivalent protein to the respective RNAs. For each point on the plot, bindings were done in triplicate and each protein was assayed twice

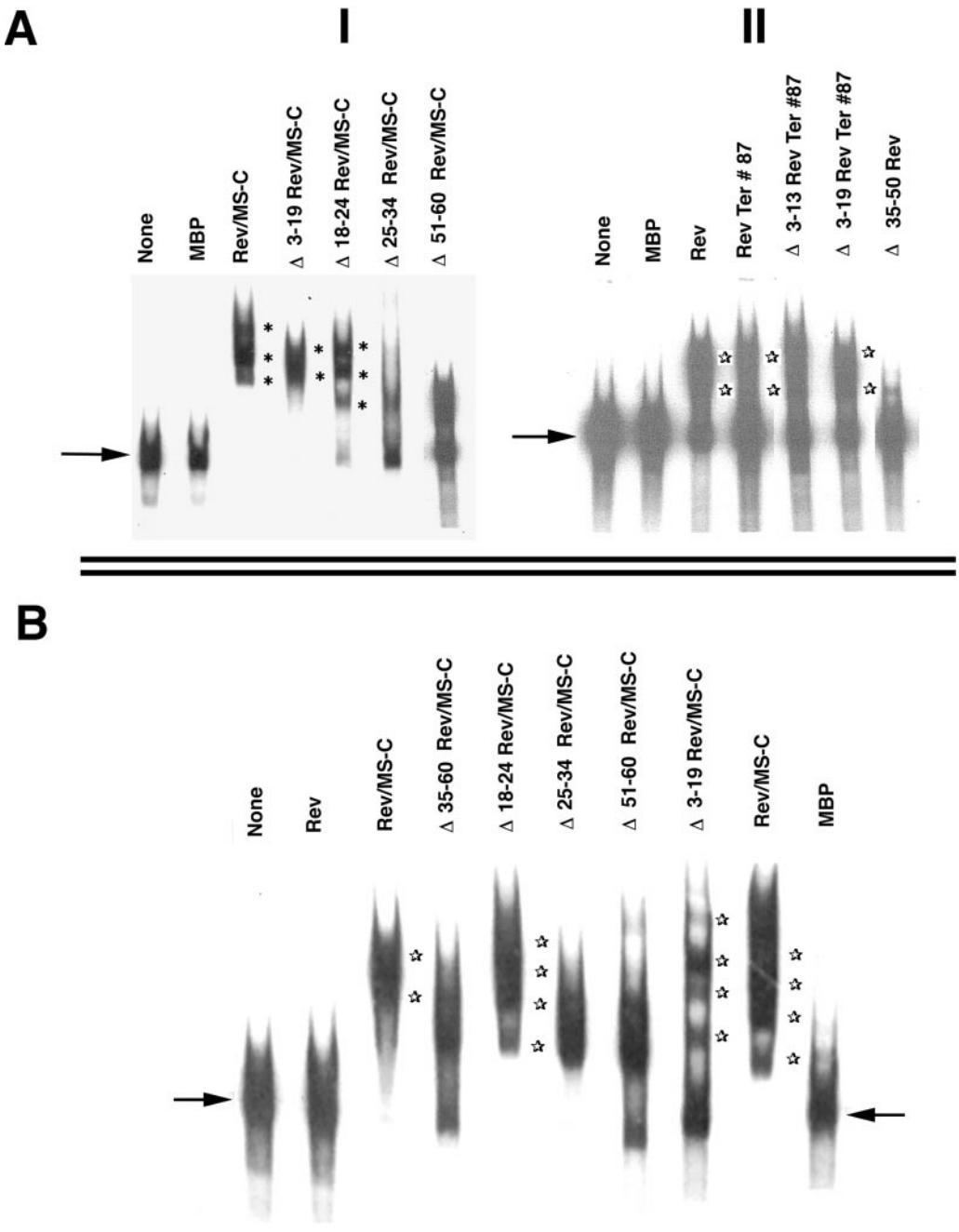


Fig. 3. EMSA of RNA binding by Rev/MS-C fusion proteins. The bacterially expressed, MBP tagged fusion proteins were purified by affinity chromatography and the MBP tags were excised by Factor Xa digestion. After purification as described under *Experimental procedures*, the individual proteins were adjusted to constant Rev equivalents. Approximately, 50 fmoles of [$\alpha-^{32}$P]-UMP labeled RRE (**A**) or RREZ-MS RNA (**B**) was incubated with the respective fusion protein in the presence of heparin and yeast tRNA and the reaction products were evaluated by EMSA. In **A**, the binding properties of Rev N-terminal deletions in the context of Rev-MS-C fusion protein or truncated Rev of 87 residues are illustrated in panels I and II. Unbound RNA is identified by an arrow. Rev and Rev/MS-C oligomers are denoted by asterisks. With Rev or Rev Ter #87 and RRE and Rev/MS-C and RREZ/MS RNA, reaction conditions favored the formation of dimers and higher order RNP complexes

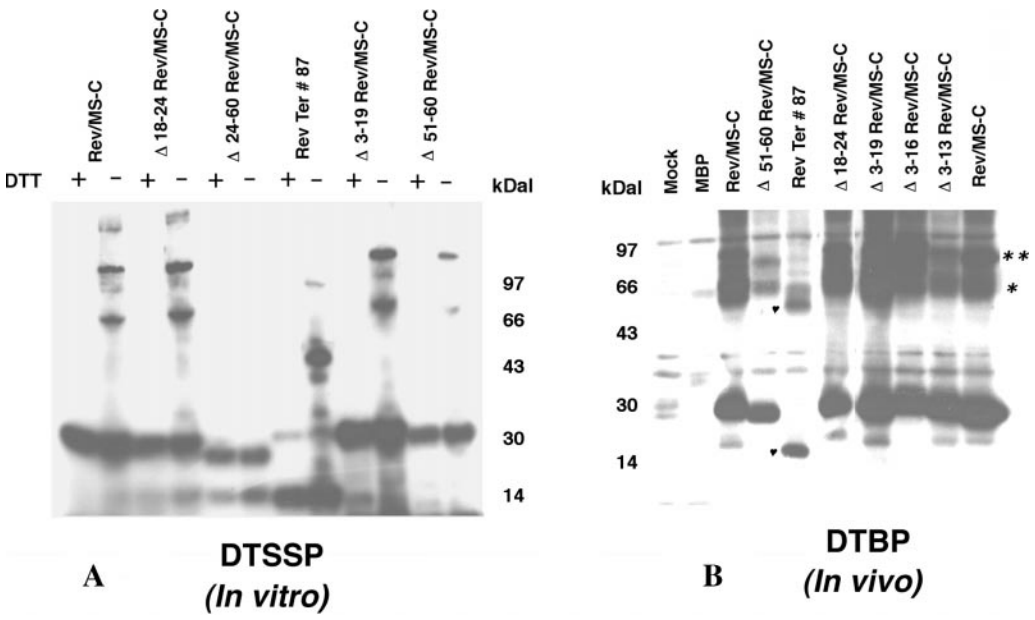


Fig. 4. Oligomerization propensities of Rev and Rev/MS-C derivatives detected by chemical cross-linking. **A** MBP tagged Rev & Rev/MS-C fusion proteins were purified by affinity chromatography. The MBP tags were removed by digesting with Factor Xa and the respective Rev and Rev/MS-C proteins were purified by negative selection on amylose resin followed by cationic cellulose chromatography. Purified proteins were adjusted to constant Rev equivalents and reacted with thiol reversible (DTSSP) bi-functional cross-linker in solution as described under *Experimental procedures*. DTSSP reacted products were treated with 1 M DTT (+) or left untreated (−) prior to electrophoresis. The protein bands were blotted on PVDF filters and detected by immuno-blotting with polyclonal rabbit anti-Rev antiserum followed by chemi-luminescence. **B** For evaluating Rev mediated multimerization in vivo, HeLa cells were electroporated with the respective Rev, Rev/MS-C derivatives. The transfectants were treated with the bi-functional chemical cross-linker, DTBP and processed for SDS/PAGE and immuno-blotting as described under *Experimental procedures*. Oligomer Rev/MS-Cs are identified by asterisks; the smaller monomer and dimer of truncated Rev of 87 residues are denoted by filled hearts

linked to yield three species bands of ca. 66, 112, and 240 kDa, representing dimer and tertamer and higher oligomers (Fig. 4A); the oligomers disappeared on treatment with 1 M DTT (Fig. 4, lane + vs −). Rev protein truncated to 86 amino-acids yielded mostly dimers. Excision of near N-terminal sequence of Rev (Δ 3/19 Rev/MS-C and Δ 18/24 Rev/MS-C), upstream of the multimerization motif did not adversely affect multimer formation. Removal of C-terminal multimerization motif of Rev (Δ 51/60 Rev/MS-C) reduced multimer formation, while deletion of N- and C-terminal multimer motifs (Δ 24/60 Rev/MS-C) abolished protein oligomerization. For assessing multimer formation in vivo, HeLa cells transiently transfected with selected Rev/MS-C derivatives were treated with the bi-functional cross-liner, DTBP as described in *Experimental procedures*. Fusion proteins were analyzed by immuno-blotting cell extracts after SDS/PAGE under

non-reducing conditions. Rev/MS-C was readily cross-linked to generate dimers and higher oligomers while the truncated Rev of 86 residues yielded mostly dimers (Fig. 4, right panel). All the N-terminal Rev deletions upstream of the multimerization motif retained the oligomerization potential. Excision of the C-terminal multimerization motif reduced, but did not abolish in vivo oligomerization (Δ 51/60 Rev/MS-C). Excision of both the N- and C-terminal multimerization motifs of Rev resulted in the loss of oligomer formation (Nam, Y.S., unpublished data). Co-expression of Rev or Rev/MS-C responsive *gag*-RRE or *gag*-RREZMS2 mRNAs did not materially alter the self-association properties of the above Rev and Rev/MS-C proteins (data not shown).

Amino-acid residues 18 through 24, immediately upstream of the 5′ oligomerization domain of Rev are critical for trans-activation

Plasmids encoding the Rev and Rev/MS-C fusion protein derivatives were electroporated into HeLa cells. Expression of various fusion proteins was monitored by immuno-blotting the extracts of transfected cells with anti-Rev anti-serum. No obvious differences in the stability of the various proteins (not shown). Sub-cellular localization of individual fusion proteins was visualized by immuno-fluorescence microscopy. As shown in Fig. 5, deletion of residues 3–13, 3–16, 3–19, or 18–24 did not alter the Rev like behavior of the respective fusion proteins localizing in the nuclei and concentrating in the nucleoli. In contrast, deletion at the RNA

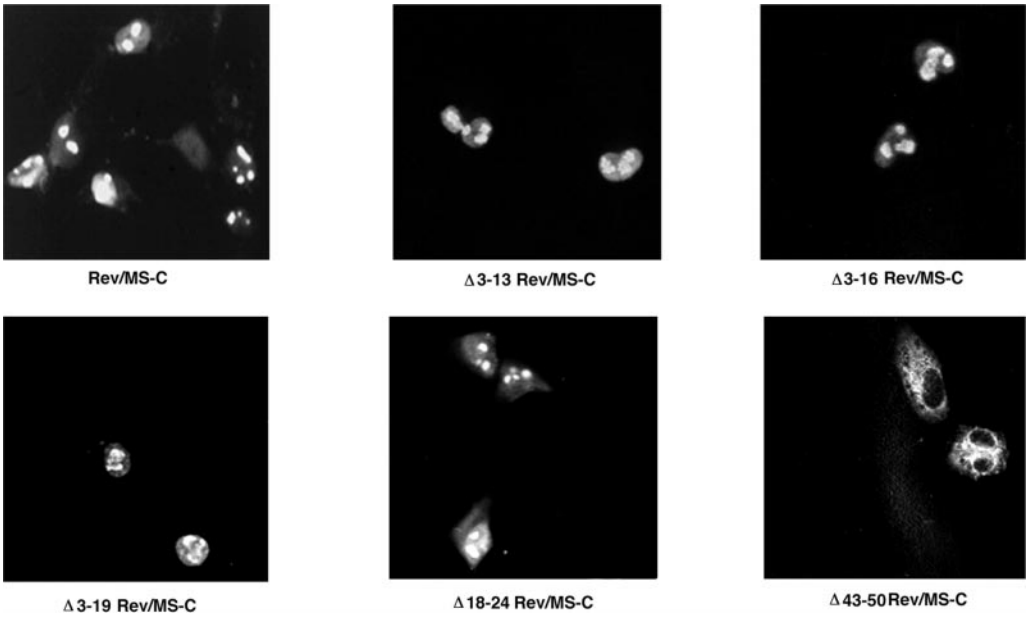


Fig. 5. Sub-cellular distribution of N-terminal deletion mutants of Rev detected by immuno-fluorescence microscopy. The indicated expression plasmids encoding the various fusion proteins were electroporated into HeLa cells and the transfectants were processed for immuno-fluorescence microscopy using a mono-clonal antibody against Rev as described in *Experimental procedures*

Table 1. *Trans*-activation potential of REV/MS-C proteins with deletions near the N-terminus of Rev for the RRE and MS2 targets

Rev and Rev/MS-C fusion proteins	trans-activation[a]	
	RRE	RREZ-MS2
REV	100	1.3 (0.7)
REV/MS-C	122 (13.7)	142 (18.2)
Δ 3/13 REV/MS-C	89 (10.7)	77 (9.8)
Δ 3/16 REV/MS-C	72 (5.4)	106 (12.3)
Δ 3/19 REV/MS-C	57 (8.5)	82 (11.2)
REV Ter #87	67 (9.1)	0.8 (0.7)
Δ 3/19 REV Ter #87	51 (7.5)	2.3 (1.1)
Δ 18/24 REV/MS-C	2.8 (1.5)	1.1 (18.3)

[a]Mean GAG expression values from six experiments, normalized for transfection efficiency are tabulated with the respective standard deviations (SD) in parentheses are shown. The positive values are denoted by bold font

binding domain of Rev (residues 43–50) caused the resulting fusion protein to be distributed diffusely in the cytoplasm.

GAG expression in the transfectants was measured by ELISA and presented in Table 1. GAG expression from plasmids containing wild type RRE was compared with expression from RREZ-MS in transfected HeLa or Cos-7 cells. For quantifying the magnitude of Rev response, extracts of cells expressing wt Rev and gag RNA were serially diluted to obtain GAG ELISA OD units of between 1 and 2. Under these conditions, positive Rev response in different experiments varied between 200 and 300 serial dilutions. The cut-off value (recommended by the manufacturer) for the negative response was at ELISA readings that fell below 10% of the control gag-RRE/REV values after the respective dilution for the experiment. ELISA readouts of undiluted or serially diluted extracts of non-transfected cells and transfectants expressing RRE mutants non-responsive to Rev fell below the 10% values of undiluted or serially diluted extracts of Rev/RRE cells. Values that ranged between 10–20% of that with RRE and REV were scored as marginal responders. GAG expression obtained with wt Rev (pRSV LTR Rev) was arbitrarily set to 100. Removal of Rev residues 3 through 13, 3–16 and 3–19 resulted in a modest decrease in the Rev dependent GAG expression from RRE containing mRNA. With the C-terminally truncated Rev of 87 residues (Rev Ter #87), a similar decrease in the Rev effect was observed. With MS2 RNA substrate, removal of Rev residues 3–19 resulted in a more subdued decrease in the gag expression (Table 1). Deletion at the RNA binding domain (residues 43–50) of Rev that caused the corresponding fusion proteins to be retained in the cytoplasm (Fig. 5) resulted in the loss of Rev activation of transcripts containing either target. Interestingly, fusion protein mutant deleted for residues 18–24 also displayed a null phenotype for both targets. This mutant did not alter the nuclear localization pattern of wt Rev (Fig. 5), retained the binding specificity for both

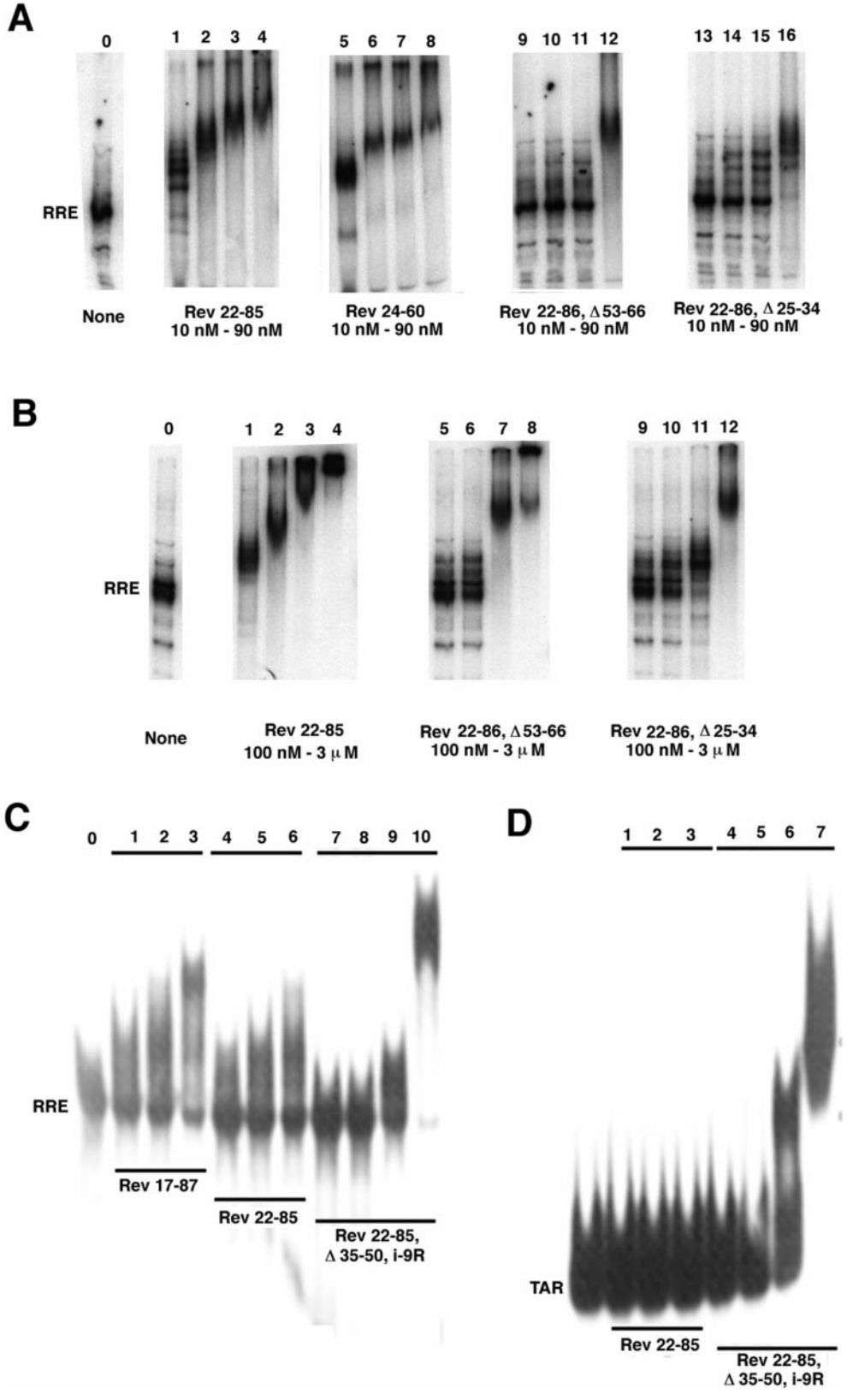
RNAs (Fig. 2), and was multimerization competent (Figs. 3 and 4). Its phenotype was quite unlike those of mutants devoid of N- (residues 25–34) or C-terminal (residues 51–60) oligomerization domains that resulted in a severe reduction or loss of GAG expression from RRE, but not MS2 RNA containing transcripts (Nam et al., unpublished data).

Rev domain peptides have reduced affinity for RRE RNA binding in vitro, yet retain substantial trans-activation potential in vivo

To precisely identify the minimal Rev sequence that will faithfully reproduce the sensitivity and the specificity for RRE binding in vitro and *trans*-activation in vivo, we synthesized peptides spanning residues 17–87, 22–85 of Rev. In addition other peptides spanning residues 22–86, but lacking either one or both oligomerization domains (residues 25–34 and 53–66) of Rev were also synthesized. To analyze the RNA binding specificity, the arginine rich RNA binding, NLS and NOS domains of Rev (residues 35–50) were exchanged for a string of nine arginines in the 22–85 peptide. A smaller peptide (residues 24–60) lacking the effector domain of Rev was used as a control for in vitro RNA binding and in vivo Rev assays.

RNA binding specificity of various Rev peptides was analyzed in vitro by EMSA. Peptides were reacted with RRE RNA in the presence of non-specific competitor tRNA. With 0.5 μg of yeast tRNA as a competitor, wt Rev peptide spanning residues 22–85 (Rev 22–85) bound RRE RNA efficiently at 10 nM; higher peptide inputs led to the formation of sequentially higher order complexes (Fig. 6A). A smaller peptide (Rev 24–60) that lacked the effector domain also behaved in analogous manner (Fig. 6A, panel Rev 24–60). Under the same conditions, mutant peptides that lacked the N- or C-terminal oligomerization domains were ten-fold less efficient for RRE binding (Fig. 6A, panels Rev 22–86, Δ 53–66 and Rev 22–86, Δ 25–34). When RRE binding was done in the presence of vast excess of tRNA (5 μg), a similar ten-fold reduction in the binding affinity was observed for the deletion mutants (Fig. 6B). Unlike the wt Rev peptides which formed a

Fig. 6. EMSA of HIV-1 RRE or TAR RNA binding by selected Rev peptides, and purified Rev and Tat proteins in the presence of non-specific competitor yeast tRNA. Reaction conditions are described in greater detail under *Experimental procedures*. **A** EMSA profile of RNA:peptide binding reactions in the presence of 0.5 μg of yeast tRNA. 100 fmoles of [$\alpha-^{32}$P]-UMP labeled RRE RNA was reacted with 10, 20, 50, or 90 nM (left to right for each panel) concentrations of the respective peptide. The different peptides used are denoted under each panel. **B** Results of RNA:peptide binding in the presence 5 μg of yeast tRNA. Peptide concentrations were 100 nM, 500 nM, 1 μM and 3 μM (left to right). **C** Comparison of RRE RNA binding by two wt (Rev 17–87 and Rev 22–85) and a nine arginine substituted (22–85, Δ 35–50, i-9R) peptide. Binding reactions were performed in the presence 0.1 μg of yeast tRNA using 10, 20, 50, or 90 nM of each peptide (left to right). **D** Comparison of HIV-1 TAR RNA binding by the wt and nine arginine substituted peptides. 200 fmoles of [$\alpha-^{32}$P]-UMP labeled TAR RNA was used. Other conditions are the same as for **C**


A
0
1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16
RRE
None
Rev 22-85
10 nM - 90 nM
Rev 24-60
10 nM - 90 nM
Rev 22-86, Δ53-66
10 nM - 90 nM
Rev 22-86, Δ25-34
10 nM - 90 nM
B
0
1 2 3 4
5 6 7 8
9 10 11 12
RRE
None
Rev 22-85
100 nM - 3 μM
Rev 22-86, Δ53-66
100 nM - 3 μM
Rev 22-86, Δ25-34
100 nM - 3 μM
C
0 1 2 3 4 5 6 7 8 9 10
RRE
Rev 17-87
Rev 22-85
Rev 22-85,
Δ35-50, i-9R
D
1 2 3 4 5 6 7
TAR
Rev 22-85
Rev 22-85,
Δ35-50, i-9R

discrete set of RRE RNA:protein complexes of increasing size, the mutant peptides with deletions of the N- or the C-terminal multimerization motifs formed a single major RNP complex. In the presence of competitor tRNA, no significant binding to the SLIIB core Rev binding site of RRE was demonstrable (data not shown). When the binding of wt Rev peptides to different RNA targets was analyzed in the presence of tRNA, RNA:peptide complexes were formed only with RRE (Fig. 6C, lanes Rev 17–87, and Rev 22–85), but not with TAR (Fig. 6D, lanes Rev 22–85) RNA. This stringent binding specificity was reduced when the RNA binding domain of wt Rev peptide was exchanged for nine arginines in the Rev 22–85 Δ 35–50-i-9R peptide. This 9 arginine peptide bound HIV-1 RRE (Fig. 6C), TAR (Fig. 6D) and MS2 (not shown) RNAs as well. From analysis of steady-state binding data generated by EMSA, K_D values in the range of 10^{-8} to 10^{-9} were calculated for wt Rev peptide binding that were an order of magnitude higher than the corresponding K_D for Rev protein. However, our real-time kinetic analysis of RRE RNA binding to Rev peptides suggested that the relatively fast dissociation of peptides from RRE precluded precise deduction of overall affinity parameters [57].

For measuring the *trans*-activation potential of peptides, Cos-7 cells were transfected by the calcium phosphate method with HIV-1 LTR linked *gag* expression plasmids containing HIV-1 RRE, RREZ-MS2, HTLV-I RexRE, or HIV-1 TAR RNA sequence embedded downstream of the *gag* transcriptional unit. CMV promoter linked HIV-1 Tat expression plasmid was co-transfected to facilitate transcription of HIV-1 LTR linked plasmids. To normalize for transfection efficiency, HIV-1 LTR linked CAT gene was also included. Rev protein or the indicated peptides were electroporated in triplicate into the above set of transfectants. Cells were harvested and processed for measurement of GAG by ELISA at 12 h following peptide or protein electroporation. In separate experiments, RSV LTR linked HIV-1 Rev, HTLV-I Rex or Rev/MS-C fusion protein plasmids were co-transfected with the respective set of *gag* expression and normalization plasmids. These control transfectants were harvested at 36–48 h later for measuring GAG expression. GAG expression values were adjusted for transfection efficiency by normalizing for CAT expression. Values obtained with the different combinations of protein(s), plasmid, or peptides and *gag* mRNAs are summarized in Table 2. For comparison, gag-RRE activation by Rev was arbitrarily assigned a value of 100. Plasmid expressed Rev/MS-C fusion protein activated both RRE and MS2 targets, and HTLV-I Rex protein activated RRE target modestly. Bacterially expressed Rev protein was quite competent for *trans*-activation of RRE containing transcripts, reaching a plateau of 88% of values obtained with plasmid expressed Rev. Native Rev peptides spanning residues 17–87 or 22–85 were somewhat less proficient, at about 70% of values obtained with plasmid expressed Rev. Higher inputs of Rev protein or peptide were somewhat toxic and failed to elicit a corresponding increase in GAG expression. As expected from the binding data, neither the Rev protein nor the wt peptides activated heterologous targets. Mutant peptides lacking one or both oligomerization motifs were uniformly negative in this assay.

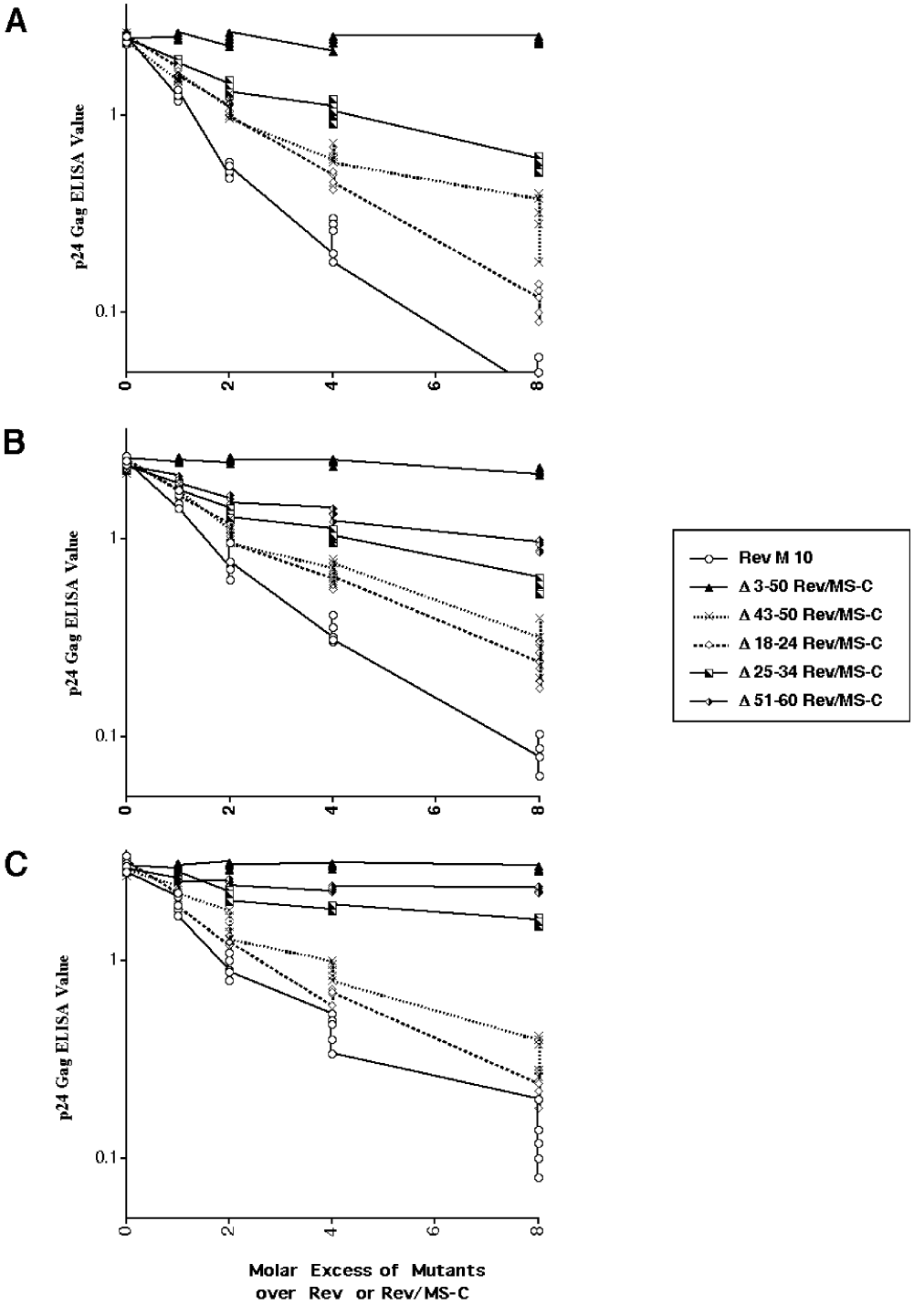
Table 2. Trans-activation potential of synthetic Rev peptides with different RNA targets

Rev fusion proteins or peptides	*trans*-activation of gag plasmids containing respective targets			
	RRE	RREZ-MS2	RexRe	Tar
pRSV-Rev	100	1.8 (0.9)	2.2 (1.4)	2.6 (0.9)
pRSV-Rev/MS-C	123 (14.4)	118 (9.2)	1.4 (0.8)	3.1 (1.2)
Rev Protein (2.5 μg)	58 (8.4)	1.3 (0.9)	2.1 (1.5)	0.8 (0.6)
Rev Protein (10 μg)	88 (12.3)	3.8 (1.8)	2.3 (1.4)	0.6 (0.5)
pRSV-Rex	43 (8.4)	1.8 (1.2)	168 (11.9)	2.3 (1.2)
Rev 22–85 (1.6 μg)	22 (4.3)	3.7 (2.4)	4.1 (2.2)	3.1 (1.9)
Rev 22–85 (3.6 μg)	41 (8.2)	5.7 (2.8)	1.7 (1.4)	0.9 (0.6)
Rev 17–87 (1.6 μg)	38 (6.2)	3.8 (2.4)	1.8 (1.2)	2.4 (1.1)
Rev 17–87 (3.6 μg)	68 (9.6)	6.1 (2.8)	1.8 (1.4)	2.1 (1.2)

N-terminal deletion mutants of Rev differentially inhibit activation of RRE and MS2 RNAs by Rev or Rev/MS-C fusion protein

We have shown that deletion of residues 18–24 abolished the Rev response irrespective of the target. But, a Rev mutant deleting residues 3–19 and a Rev peptide spanning residues 22–85 retained partial Rev function in vivo. Since deletion of residues 18–24 did not perturb specific RNA binding, we reasoned that these residues may be critical for other aspects of Rev function in vivo. If it were so, this mutant may interfere with wt Rev function.

HeLa cells were co-transfected with expression plasmids for Rev or Rev/MS-C and GAG indicator plasmids incorporating RRE or MS2 targets together with increasing amounts of different Rev or Rev/MS-C mutant plasmids. The well studied trans-dominant mutant M10 at the effector domain of Rev was used as a positive control for inhibitory response. A Rev/MS-C deletion mutant (Δ 3/50 Rev/MS-C) lacking residues 3–50 of Rev in the context of the Rev/MS-C fusion protein served as a negative control. Rev dependent expression of GAG protein was determined by ELISA and normalized for transfection efficiency as described under *Experimental procedures*. Normalized GAG ELISA values are plotted (on semi-log scale) as a function of inhibitor plasmid input in Fig. 4. Absolute values of individual plots from four separate experiments were factored to be within the same range of GAG expression. As expected, the M10 mutant inhibited GAG expression from Rev:*gag*RRE, (Fig. 7A), Rev/MS-C:*gag* RRE (Fig. 7B), and Rev/MS-C:*gag*RREZ/MS2 (Fig. 7C) in a dose dependent manner. Under the same conditions, the Δ 3/50 Rev/MS-C mutant had no effect on the Rev response. A deletion mutant (Δ 43/50 Rev/MS-C) that excised a portion of the RNA binding domain of Rev in the context of the Rev/MS-C fusion protein also inhibited the Rev response of both RRE and MS2 targets. Two deletion mutants that excised the N-terminal (Δ 25/34 Rev/MS-C) or the C-terminal (Δ 51/60 Rev/MS-C) oligomerization motifs of Rev induced a less profound inhibition of GAG expression in response to Rev (Fig. 7A) or Rev/MS-


A
B
C
p24 Gag ELISA Value
1
0.1
0
2
4
6
8
Molar Excess of Mutants over Rev or Rev/MS-C
Rev M 10
Δ 3-50 Rev/MS-C
Δ 43-50 Rev/MS-C
Δ 18-24 Rev/MS-C
Δ 25-34 Rev/MS-C
Δ 51-60 Rev/MS-C

C (Fig. 7B). However, our multimerization mutants were not very efficient at inhibiting GAG expression from mRNAs containing MS2 RNA target (Fig. 7C), implying that Rev multimerization may only be required for activation of RRE and not heterologous RNAs (Nam et al., unpublished data). The mutant that deleted residues 18–24 of Rev (Δ 18/24 Rev/MS-C) was uniformly inhibitory for GAG expression induced by Rev or Rev/MS-C proteins. Δ 18/24 Rev/MS-C that accumulated exclusively in the nucleus (Fig. 5) and had no known defects in RNA binding or protein multimerization thus represents a novel class of *trans*-dominant repressor.

Discussion

Our studies have shown that a minimal Rev sequence spanning residues 19–87 retained the specificity of native Rev for *trans*-activation of RRE RNAs. Excising the N-terminal residues 3–19 of Rev in the context of the Rev/MS-C fusion protein spared the RNA binding specificity and the activation potential for both RRE and MS2 *gag* mRNAs. These findings were further supported by the conserved fidelity of activation or RRE transcripts by wt Rev peptides spanning residues 22–85. Peptides spanning residues 17–87 or 22–85 bound RRE RNA specifically in vitro, but at a ten-fold reduced affinity when compared with Rev. Smaller peptides, lacking one or both members of the bi-partite oligomerization motif exhibited five to ten-fold less affinity for RRE RNA than wt peptides. In the absence of competing RNA, the wt, smaller peptides or the nine arginine substituted derivatives were uniformly unable to discriminate RRE RNA from other unrelated RNAs. This behavior is similar to that reported by Daly et al. [10] who examined the RNA binding characteristics of a Rev peptide, RP194 spanning residues 17–58, a truncated Rev protein lacking twenty N-terminal residues, and the M4 oligomerization mutant [34, 35, 38] with changes at residues 23, 25, and 26. Real-time kinetic analysis of RRE binding by the Rev peptide(s) spanning 22–85 and 17–87 [57] also appeared to support this conclusion. However, as pointed out in [57], the Rev domain peptides displayed unusually fast dissociation from immobilized RRE RNA precluding precise determination of steady-state kinetics. We have shown that by carefully titrating the amounts of competing tRNA and peptide, highly specific interactions with RRE RNA can be obtained. However, the affinity of the

Fig. 7. Rev N-terminal deletion mutants inhibit activation of RRE or MS2 targets by wt Rev or Rev/MS-C differentially. **A** shows the effects of mutants on RRE activation by RSV LTR linked Rev; **B** the effects on RRE activation by RSV LTR linked Rev/MS-C; and **C** on MS2 activation by RSV LTR linked Rev/MS-C. Transcription from HIV-LTR linked gag-RRE or gag-MS2 expression target plasmids was maintained by co-transfection CMV promoter linked HIV-1 Tat plasmid. Luciferase expression from HIV LTR linked luciferase plasmid was used to normalize for transfection efficiency. Increasing amounts of the RSVLTR linked Rev M10, Δ 3/50 Rev/MS-C, Δ 18/24 Rev/MS-C, Δ 25/34 Rev/MS-C, Δ 43/50 Rev/MS-C, and Δ 51/60 Rev/MS-C mutants that were used are identified by the respective symbols in the figure

peptide for RRE RNA was ten-fold less than that for Rev. This may suggest that truncated Rev peptides cannot discriminate between RRE and unrelated RNAs. Even peptides that lacked the 5′ (Rev 22–86, Δ 25-34) or 3′ (Rev 22–86, Δ 53–66) oligomerization domains of Rev possessed residual specificity for RRE RNA; however their respective affinities for RRE RNA were considerably less than those of wt. RNA binding by shorter peptides that lacked both domains was below this threshold of binding specificity. In light of these observations, we like to suggest that a 17 mer RNA binding domain (residues 35–50) of Rev is sufficient for the initial "searching and discriminatory" process that leads to docking on a specific RNA site as was suggested before [7, 30, 31, 54]. Changes in this core binding domain will result in the loss of discrimination of RNA; appending the correct Rev sequence imparting alpha helicity [2, 3, 54, 55] may increase the affinity for the target by reducing the rate of dissociation of the bound peptide [10, 57] and permitting higher-order protein:protein interactions at the binding site. On the contrary, appending an inappropriate sequence to the core peptide may have the opposite effect of enhancing the dissociation of the bound peptide. For instance, addition of upstream Tat sequence to a basic domain peptide of Tat reduced the in vitro affinity for TAR RNA [47].

Removal of residues 18–24 of Rev in the fusion protein abolished Rev phenotype for both targets. Although this mutant probably had no defects in the rates of nuclear shuttling, it is possible that deleting Rev residues 18–24 may have impaired the steady-state kinetics (i.e. fast off rates) of RRE RNA. Alternatively, the 18–24 domain that has not been directly implicated in Rev multimerization, may still be crucial as part of the recently proposed "hydrophobic surface patch" [56] facilitating intermolecular interactions. But, deleting the 18–24 Rev sequence in the context of the Rev/MS-C fusion protein also impaired *trans*-activation of MS2 RNA, Binding to the MS2 RNA is mediated by the MS2 coat protein moiety [41, 58], and activation of MS2 RNA does not require the presence of Rev oligomerization domain(s) (Nam YS et al., unpublished data). Therefore, and as discussed below, the 18–24 sub-domain of Rev may be critical for cellular protein binding, or facilitate proper folding of Rev to permit such cellular protein interactions.

Several inhibitory Rev mutants have been identified. Of these, the C-terminal effector domain mutants, notably, M10 [35] have been the best characterized. M10 and other mutants of that genre that bind RRE RNA, but fail to interact with the host nuclear export factor, CRN-1 competitively inhibit wt Rev function [4, 11, 35, 40]. Among the N-terminal Rev mutants, two classes have been described. A class of mutants with defects in Rev multimerization, but without obvious defects for RRE RNA binding have been shown to inhibit wt Rev, presumably by sequestering all the available RRE RNA to the exclusion of wt Rev [56]. Mutants that delete part or all of the RNA binding motif (which also serves as the NLS and NOS) have been shown to inhibit the nuclear and nucleolar accumulation of Rev, presumably by sequestering wt Rev in the cytoplasm [32, 33]. Our representatives for each of the above classes, Δ 43/50 Rev/MS-C mutant deleting the NLS/NOS, and the Δ 25/34 Rev/MS-C, and Δ 51/60 Rev/MS-C mutants

deleting the N- or the C-terminal oligomerization motifs had the expected inhibitory phenotype for RRE activation by Rev or Rev/MS-C. However, mutants deleting the oligomerization domain were not efficient inhibitors of Rev (as the Rev/MS-C fusion protein) mediated activation of the heterologous MS2 target, implying a target specific multimerization requirement for Rev function. In addition to the above two classes of mutants, we have identified another inhibitory mutant, mapping to residues 18–24, that is mechanistically different from the rest. Based on the peptide binding data, this deletion mutant may be expected to have reduced affinity for RRE RNA. But, the Δ 18/24 Rev/MS-C mutant had no obvious defects in RNA binding, nuclear accumulation, and oligomerization. Its null phenotype for MS2 targets suggests that Rev residues 18–24 may be necessary for functions other than stabilizing RRE RNA complex. Unlike the M4 substitution mutant that changed the -YQSN- sequence to -DGDL- between residues 23–26 with consequent loss of multimerization [10, 34, 35, 38] and discriminatory RNA binding [10], deleting residues 18–24 in the context of Rev protein did not eliminate the multimer formation in vitro. The inhibition of Rev mediated activation of RRE by Δ 18/24 Rev/MS-C may have been due to formation of unproductive hetero-oligomer complexes with wt Rev. Since Rev multimerization is not required for MS2 RNA binding and since the mutant binding to wt Rev/MS-C should not interfere with MS2 RNA binding by the coat protein moiety, *trans*-dominant inhibition of MS2 targeted activation strongly implies that residues 18-24 are critical for other events following RNA binding.

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Authors' address: Dr. S. Venkatesan, LMM, NIAID, Building10, Room 6A05, National Institutes of Health, Bethesda, MD, 20892-1576, U.S.A.

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